2-22-00

PATENT
454311-2200.1

jc604 U.S. PTO
02/18/00

Patent Application Transmittal (for new nonprovisional appln under 37 C.F.R. 1.53(b))

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jc525 U.S. PTO
09/507242
02/18/00

Date: February 18, 2000 Re: 454311-2200.1

TO: ASSISTANT COMMISSIONER FOR PATENTS
Box NEW PATENT APPLICATION, Washington, D.C. 20231
Sir: With reference to the filing in the United States Patent and Trademark Office of an application for patent in the names of: **HANES, Steven D.**, **DEVASAHAYAM, Gina** and **CHATURVEDI, Vishnu** (information on attached, unsigned, Declaration & Power of Attorney), and entitled:

***CaESSI*: A CANDIDA ALBICANS GENE, METHODS FOR MAKING AND USING, AND TARGETING IT AND ITS EXPRESSION PRODUCTS FOR ANTIFUNGAL APPLICATIONS**

Enclosed is:

X Specification (41 pages, plus 1 page of abstract, p. 45)
X 5 Sheet(s) of Drawings (Figs 1A, 1B, 2, 3, 4)
X 23 Claims (pp. 42-44)
X Oath or Declaration and Power of Attorney (3pp UNSIGNED)
X The filing fee will be paid and an executed Declaration and Power of Attorney will be filed upon receipt of a Notice To File Missing Parts. Kindly accord the application a February 18, 2000 filing date.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP
Attorneys for Applicants

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EXPRESS MAIL
Mailing Label Number: EL197532271US
Date of Deposit: February 18, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, BOX NEW PATENT APPLICATION.

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TITLE OF THE INVENTION

CaESS1: A *CANDIDA ALBICANS* GENE, METHODS FOR MAKING AND USING, AND TARGETING IT OR ITS EXPRESSION PRODUCTS FOR ANTIFUNGAL APPLICATIONS

STATEMENT OF GOVERNMENT INTEREST

Without any prejudice or admission, this invention may have been made with funding from the National Institutes of Health, HRI grant # 815-3487, such that the U.S. Government may have certain rights.

RELATED APPLICATIONS

This application claims priority from U.S. Application Ser. No. 60/121,246, filed February 23, 1999. U.S. Application Ser. No. 60/121,246, each document cited therein ("USSN 60/121,246 cited documents"), and each document referenced or cited in USSN 60/121,246 cited documents are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to compositions and methods for diagnosing and/or detecting and/or preventing and/or treating *Candida albicans* or conditions or symptoms associated therewith, as well as to process and products for preparing such compositions and methods.

The present invention further relates to *CaESS1,* an important *Candida albicans* gene, e.g., nucleic acid molecules therefor, and/or fragments or portions thereof, expression products therefrom, e.g., the protein CaEss1 or fragments or portions thereof, methods for making and using the gene, portions thereof and expression products therefrom, and to targeting the gene or portions thereof and/or the expression products therefrom for antifungal applications.

The identification of the *CaESS1* gene allows for identifying compounds or agents that specifically bind to and/or inhibit the gene, or portions thereof and/or expression products therefrom, and methods for preventing and/or treating *Candida albicans* and/or symptoms or conditions associated therewith, as well as generally for making and using such compounds or agents. Thus, the invention relates to antifungal preparations and/or compositions and methods for making and using them.

The CaEss1 amino acid sequence and the *CaESS1* DNA or nucleic acid sequences can be used for diagnostic purposes. For instance, the nucleic acid sequences can be used to generate primers for diagnostic DNA, and the invention comprehends such primers. Primers are preferably derived from those parts of the *CaESS1* gene which are least conserved among the *ESS1/PIN1* family members. The gene or the primers can be used to detect if the gene is present in a sample or specimen and/or if the gene was expressed as RNA in a sample or specimen. Accordingly, the invention relates to compositions and methods for detecting and/or diagnosing *Candida albicans*.

The *CaESS1* gene and portions thereof are useful for generating or expressing the CaEss1 protein and epitopic portions thereof (epitopic portions of the protein can be derived from, generated by, or expressed from those parts of the *CaESS1* gene which are least conserved among the *ESS1/PIN1* family members). The protein or portions thereof is useful for generating antibodies, such as monoclonal and/or polyclonal antibodies. These antibodies can be used for diagnostic purposes; and, the protein or portions thereof can be used for diagnostic purposes, e.g., the antibodies can be used to detect or determine, e.g., via binding, whether the proteins or portions thereof are present in a sample or specimen and the protein or portions thereof can be used to detect or determine, e.g., via binding, whether antibodies thereto are present in

a sample or specimen. Further, the antibodies can be used to block CaEss1 activity. Accordingly, the invention relates to diagnostic compositions and methods, as well as therapeutic or preventive compositions and methods.

The invention further relates to methods for screening compounds for the ability to inhibit CaEss1 and/or PIN1. Compounds which selectively inhibit CaEss1 and do not inhibit PIN1 or do not inhibit PIN1 greatly are compounds useful in the prevention and/or treatment of *Candida albicans*. Compounds which inhibit PIN1 are useful in antiproliferative applications, e.g., as antineoplastic, anti-tumor or anticancer agents. Furthermore, the screening methods of the invention can be adapted and used for screening compounds which inhibit other fungal infection as fungus other than *Candida albicans* have *ESS1* genes. Accordingly, the invention relates to methods for screening for inhibitors of CaEss1, PIN1 or other ESS1s, as well as to inhibitors of these enzymes.

Various documents are cited in this text, or in a reference section preceding the claims. Each of the documents cited herein, and each of the documents cited or referenced in each of those various documents, is hereby incorporated herein by reference. None of the documents cited in the following text is admitted to be prior art with respect to the present invention.

BACKGROUND OF THE INVENTION

Candida albicans is an asexual yeast species. *Candida albicans* is a major fungal pathogen of humans. It is can be found as a harmless commensal organism, inhabiting mucosal membranes and the digestive tract; a benign saprophyte. However, *Candida albicans,* can infect both internal organs and mucous membranes of the mouth, throat, and genital tract, and can cause a chronic infection; it can cause

superficial infections, such as oral thrush, and can cause severe, often fatal, systemic infections, especially in immunocompromised patients.

There has been a growing number of cases of thrush and other diseases caused by *Candida albicans*; and, this can be attributed mainly to medical advances in antibiotic, steroid and immunosuppressive treatments, as well as to immunocompromising ailments such as HIV and AIDS. Indeed, surveillance of nosocomial blood stream infections (BSI) in the USA between April 1995 and June 1996 revealed that *Candida albicans* was the fourth leading cause of nosocomial BSI (Pfaller et al., "National surveillance of nosocomial blood stream infection due to *Candida albicans*: frequency of occurrence and antifungal susceptibility in the SCOPE Program," *Diagn Microbiol Infect Dis* 1998 May;31(1):327-32). Accordingly, *Candida albicans*, and compositions and methods for detecting, diagnosing, preventing or treating *Candida albicans* are medically significant.

Thrush is characterized by creamy-white, curdlike patches on the tongue and other mucosal surfaces of the mouth. The disease is caused by an overgrowth of *Candida albicans*. Patients susceptible to thrush include immunocompromised individuals, e.g., adults whose immune systems have been weakened by antibiotics, steroids, immunosuppression treatments, AIDS, and the like, as well as infants, for instance if the mother had a vaginal yeast infection.

Painful, raw and bleeding areas result if the curdlike discharge is removed from patches of thrush. These superficial lesions may allow the yeast to spread to other areas of the body. *Candida albicans* can invade major organs, causing serious complications.

While thrush is typically treated with a topical agent, and there are oral and intravenous treatments for *Candida albicans* infections, chronically infected patients may require long term therapy with oral and/or intravenous therapy.

Moreover, strains of *Candida albicans* resistant to present treatments or therapies such as amphotericin B, fluconazole, itraconazole and other azole antifungals have been isolated (Mori et al., "Analysis by pulsed-field gel electrophoresis of *Candida albicans* that developed resistance during antifungal therapy," *Nippon Ishinkin Gakkai Zasshi* 1998;39(4):229-33; Pfaller et al., *supra*; Rex et al., "A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia," *N Engl J Med* 1994 Nov 17;331(20):1325-30). Indeed, in Rex et al., in certain individuals, treatment failed to clear infection from the bloodstream, and *Candida albicans* was infection commonly associated with the treatment failure.

Thus, there is a need for new treatments or therapies against *Candida albicans.*

Diagnosis of *Candida albicans* requires microscopic identification of the *pseudomycelial* (branching-arms) forms. There is likewise a need for new compositions and methods for diagnosing or detecting *Candida albicans.*

The *ESS1* gene was originally discovered in *Saccharomyces cerevisiae*, by inventor Hanes working in the laboratory of Dr. Peter Shank and Dr. Keith Bostian (Hanes 1988). It was discovered in a search for cell growth control genes. By gene disruption techniques, *ESS1* was shown to be essential for yeast cell growth, hence the name (Essential) (Hanes et al. 1989). *ESS1* genes are highly conserved. Homologs of the *ESS1* gene have been found in *Drosophila*, humans and several other organisms.

The fly gene (called *dodo*) and the human gene (called *PIN1*) encode proteins that are 45% identical to the yeast *Essl* protein (Maleszka et al. 1996; Lu et al. 1996).

WO 97/17986 relates to the identification and characterization of Pin1, a protein of mammalian origin that associates with NIMA protein kinase. It was determined that overexpression of Pin1 activity induces a specific G2 arrest and delays NIMA-induced mitosis, while depletion of Pin1 triggers mitotic arrest and nuclear fragmentation. The specification provides for a method of controlling the growth of a cell by contacting the cell with a composition which modulates the Pin1 activity. See also U.S. Patents Nos. 5,952,467 and 5,972,697.

However, prior to the present invention, the *Candida albicans ESS1* or *CaESS1* gene had not been isolated, or sequenced; or disclosed or suggested, nor had corresponding amino acid sequences from the gene been disclosed or suggested. Likewise, fragments or portions of the gene and protein had not been disclosed or suggested. Also, diagnostic, prophylactic, therapeutic, or similar compositions and methods involving the gene and/or the protein and/or fragments of the gene and/or fragments of the protein, had not been taught or suggested.

In view of the significance of *Candida albicans*, and the need for new therapies, treatments, means for prevention, and means for diagnosing or detecting *Candida albicans*, providing the *CaESS1* gene, portions thereof, amino acid sequences from the gene, fragments or portions of the protein, and diagnostic, prophylactic, therapeutic, or similar compositions and methods involving the gene and/or the protein and/or fragments of the gene and/or fragments of the protein, are significant advances in the art, addressing problems in the art.

OBJECTS AND SUMMARY OF THE INVENTION

An object of the invention can include providing any or all of: the *CaESS1* gene, portions thereof, amino acid sequences from the gene, fragments or portions of the protein, and diagnostic, prophylactic, therapeutic, or similar compositions and methods involving the gene and/or the protein and/or fragments of the gene and/or fragments of the protein.

Accordingly, the present invention provides an isolated and/or purified nucleic acid molecule encoding CaEss1, e.g., *CaESS1*; for instance, an isolated and/or purified nucleic acid molecule comprising a nucleotide sequence encoding CaEss1 as set forth in Figure 1 (SEQ ID NO: 1).

The present invention also provides an isolated and/or purified nucleic acid molecule which is a primer for an isolated and/or purified nucleic acid molecule encoding CaEss1, e.g., *CaESS1,* for instance, a primer for an isolated and/or purified nucleic acid molecule comprising a nucleotide sequence encoding CaEss1 as set forth in Figure 1. Such a primer can be OW-216 or OW-221 disclosed below (SEQ ID NOS: 3, 6).

The present invention also provides an isolated and/or purified CaEss1 protein; for instance, such a protein from expression of any or all of the foregoing nucleic acid molecules, or as shown in Figure 1 (SEQ ID NO:2).

The invention further provides nucleic acid molecules and amino acid molecules having at least 70%, e.g., at least 75%, such as at least 80%, e.g., at least 85%, preferably at least 90%, more preferably at least 95% such as at least 97% homology, identity or similarity to such molecules disclosed herein.

The invention further provides diagnostic compositions and methods involving the nucleic acid molecules, as well as the amino acid molecules or antibodies generated therefrom.

Thus, the invention further provides methods for determining the presence of *Candida albicans* in a sample; for instance, by detecting the presence in the sample of *CaESS1* e.g., by diagnostic PCR using a primer or probe specific for *CaESS1*; or, by detecting CaEss1 by contacting the sample with an antibody specific for CaEss1 and detecting binding of the antibody; or by detecting antibodies to CaEss1 by contacting the sample with an inventive amino acid molecule and detecting binding thereof to an antibody in the sample.

The invention further provides therapeutic or preventive compositions, e.g., compositions useful for treating or preventing a fungal infection such as a *Candida albicans* infection or for antiproliferative effect, e.g., antineoplastic, anti-tumor or anti-cancer effect, as well as to methods for treating or preventing such fungal infections or cell proliferation.

A CaEss1 inhibitor can be a compound which selectively inhibits growth of *S. cerevisiae* not containing an endogenous *ESS1* gene but rather *CaESS1* and uninduced *PIN1* (e.g., on a glucose medium, see Figs. 2, 3) and/or preferably does not inhibit or significantly inhibit induced PIN1, e.g., does not inhibit or significantly inhibit *S. cerevisiae* not containing an endogenous *ESS1* gene but rather induced *PIN1* (see Fig. 3). An anti-CaEss1 antibody or an antibody against an epitopic region of CaEss1 can also be an inhibitor of CaEss1 by virtue of the antibody being able to bind to CaEss1. Compositions which indeed inhibit PIN1 are nonetheless useful as antiproliferatives, e.g., antineoplastics, anti-tumor agents or anti-cancer agents.

The invention comprehends methods for preventing or treating *Candida albicans* or cancer by administering the inventive compositions.

Further still, the invention provides methods for screening compounds for inhibiting *Candida albicans*, as well as other fungal infectious agents, and human cell growth. The screening method entails plating yeast transformed to express a fungal *ESS1* gene such as *CaESS1* alone or with *PIN1*, and contacting those yeast with a potential inhibitory compound, whereby compounds specifically inhibit yeast cell growth when *CaESSI* is expressed, but not when *PIN1* is expressed. Such compounds are specific inhibitors of ESS1 such as CaEss1, but not *PIN1*.

The invention yet further still entails a method for screening for antiproliferative compounds, e.g., anti-tumor, anticancer or antineoplastic agents, comprising plating yeast transformed to express low or high levels of *PIN1* (e.g., on glucose/galactose and glucose media) and determining compounds which selectively inhibit growth of yeast expressing low levels of *PIN1*, e.g., on the glucose/galactose medium, but not growth of yeast expressing high levels of *PIN1*, e.g., on the galactose medium.

The terms "comprises," "comprising," and the like can have the meaning ascribed to these terms in U.S. Patent Law and can mean "includes," "including," and the like.

These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF FIGURES

The following Detailed Description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

Figure 1A shows the complete nucleotide sequence of the *CaESS1* gene from *Candida albicans* and its predicted translation product (the *CaESS1*-encoded protein (CaEss1) is 177 amino acids long and has a predicted molecular weight of 19.8 Kd; it is 42% identical to the *ESS1* protein of *Saccharomyces cerevisiae*) (SEQ ID NOS: 1, 2);

Figure 1B shows the strategy used to delete *CaESS1* from *C. albicans* and Table 1 summarizes the results obtained, which are consistent with *CaESS1* being essential in this organism;

Figure 2 shows the strategy for identifying inhibitors specific for *Candida albicans* CaEss1 (the yeast *Saccharomyces cerevisiae* is engineered to express both the *Candida albicans CaESS1* gene and the human *PIN1* gene; the endogenous *Saccharomyces cerevisiae* gene is deleted; the *hPIN1* gene is expressed from an inducible promoter (e.g., the *GAL1* promoter) so that it can be turned off and on by changes in the culture medium (strains can also bear other changes such as mutations that favor higher efficiency drug entry or retention and the *CaESS1* gene might be modified so as to make it more sensitive to the effects of chemical inhibitors, for example, by the introduction of conditional-lethal mutations, as the use of such mutations, e.g., temperature-sensitive mutations, for screens carried out under permissive or semi-permissive conditions would sensitize cells to the effects of *CaESS1* or CaEss1 inhibitors));

Figure 3 shows a screen for *CaESS1* or CaEss1 inhibitors (cultures of specially engineered *Saccharomyces cerevisiae* (see Fig. 2) are grown in duplicate plates under different conditions, e.g., one condition is glucose, another condition is galactose (or a mixture of glucose/galactose which produces low but sufficient levels of *hPIN1* expression for cell viability, and this might be useful because massive overproduction of *hPIN1*, e.g., in galactose, might overcome compounds that inhibit both *CaESS1* or CaEss1 and *hPIN1* or PIN1, thus leading to possible false positives, i.e., possible compounds that inhibit both the fungal and human gene function); cells grown in glucose-containing medium express *CaESS1* but not *hPIN1*; cells grown in galactose-containing medium express both *CaESS1* and *hPIN1*; potential inhibitory compounds are applied to each well in duplicate, yeast growth is monitored; many compounds may have no effect, compounds in which yeast cell growth in both glucose and galactose plates (B2 and D3 in Fig. 3) inhibit both *CaESS1* or CaEss1 and *hPIN1* or hPIN1 and/or totally unrelated pathways (non-specific inhibitors of yeast cell growth). Compounds which inhibit yeast growth only in glucose plate (C5 in Fig. 3) are *CaESS1* or CaEss1-specific inhibitors; and

Figure 4 shows a screen for *hPIN1* or hPIN1 inhibitors (cultures of specially engineered *Saccharomyces cerevisiae* in which the *ESS1* gene is deleted and a *hPIN1* under the control of an inducible promoter (such as *GAL1*) is present are grown in duplicate plates under different conditions, e.g., one condition is to generate low levels of PIN1 sufficient for cell growth (e.g., glucose/galactose), another condition is to generate high levels of PIN1 (e.g., galactose); cells grown in medium containing a mixture of glucose/galactose express low levels of *hPIN1*; cells grown in galactose-containing medium express high levels of *hPIN1*; potential inhibitory compounds are applied to each well in duplicate, yeast growth is monitored; many compounds may

have no effect, compounds in which yeast cell growth in both glucose/galactose and galactose plates (B2 and D3 in Fig. 4) non-specifically inhibit yeast cell growth, and compounds which inhibit only yeast growth in glucose/galactose plate (C5 in Fig. 4) are *hPIN1* or hPIN1-specific inhibitors, as massive overproduction of *hPIN1* (in galactose) overcomes the inhibitory effect (by titrating out the inhibitor).

DETAILED DESCRIPTION

Peptidyl-prolyl *cis/trans* isomerases (PP1ases) fall broadly into three families: Cyclophilins, FKBPs and Parvulins. Parvulins, a recently discovered family, are distinct from the cyclophilins and FKBPs in both structure and substrate specificity. The Ess1 protein from *Saccharomyces cerevisiae* is a member of the parvulin-family of PPIases and is the only PPIase essential for growth in this organism. Depletion of Ess1 causes mitotic arrest and nuclear fragmentation. Homologs of the *ESS1* gene have been found in many organisms, including humans (*PIN1*), *Drosophila (dodo)*, *Aspergillus, Schizosaccharomyces* and *Neurospora*.

To explore the role of Ess1 in the biology and virulence of *Candida albicans*, Applicants sought to isolate a *C. albicans* homolog. Given the essential role of Ess1 in *S. cerevisiae*, and its high degree of structural and functional conservation, Applicants used temperature-sensitive *ess1* mutants (*ess1*^{ts}) in *S. cerevisiae*. A multicopy *C. albicans* genomic library was used to complement the growth defect of an *ess1*^{ts} mutant strain at the restrictive temperature (37 °C).

Applicants obtained 5 clones from a total of 2.0×10^6 transformants whose complementing activity was plasmid-linked. Three of the clones carried an extragenic suppressor. The other two carried an identical 8.5 kb DNA insert. Within the insert, a gene was identified encoding a 177 aa protein that is 42% identical to Ess1. Applicants call this gene *CaESS1* (see Fig. 1).

A 1kb subclone carrying only this gene was shown to complement several different *ess1*^{ts} alleles in *S. cerevisiae*. Gene knockout experiments can show *CaESS1* is an important gene in *C. albicans* and mutations in *CaESS1* affect virulence in animal models.

More generally, herein Applicants provide the discovery of a gene from an important human pathogenic fungus, *Candida albicans*, that can serve as a useful antifungal drug target. This gene, called *CaESS1*, is functional in the common laboratory yeast, *Saccharomyces cerevisiae*, that has been used extensively for high-throughput screening. The invention comprehends inhibitors of *CaESS1* or of its expressed protein CaEss1, e.g., compounds or agents which bind to either the nucleic acid molecule or the expressed protein; and, this description provides a means using *Saccharomyces cerevisiae* to carry out large screens for such inhibitors so that in view of this disclosure and the knowledge in the art, no undue experimentation is required to identify compounds or agents which so act as inhibitors. Such inhibitors can then be developed into new broad-spectrum drugs to treat patients with *Candida albicans* infections, and potentially other life-threatening fungal infections.

The *ESS1* gene was originally discovered in *Saccharomyces cerevisiae* (Hanes 1988). It was discovered in a search for cell growth control genes. By gene disruption techniques, *ESS1* was shown to be essential for yeast cell growth, hence the name (Essential) (Hanes et al. 1989).

ESS1 genes are highly conserved. Homologs of the *ESS1* gene have been found in *Drosophila*, humans and several other organisms. The fly gene (called *dodo*) and the human gene (called *PIN1*) encode proteins that are 45% identical to the yeast *Ess1* protein (Maleszka et al. 1996; Lu et al. 1996). When introduced into *ESS1* knockout strains of yeast, both the fly and human genes rescue the lethal phenotype.

These results demonstrate that these *ESS1* homologs carry out similar functions by acting on common targets within cells of the respective organism. Given that *ESS1* is highly conserved, it is likely to be present in many different pathogenic fungi, and based on its role in budding yeast, these *ESS1* homologs are likely to be essential.

Indeed, "gene knockout" results obtained by the applicants strongly suggest that the *CaESS1* gene is essential for growth in *C. albicans* just as the *ESS1* gene is essential in *S. cerevisiae*. *C. albicans* is a diploid organism and therefore should have two alleles of *CaESS1*. Experiments done by the applicants show that it is possible to delete one of the two alleles of *CaESS1* in *C. albicans* (Figure 1B). This was done by the method of homologous recombination as described by Fonzi and Irwin (1993). However, the applicants have not been able to delete the second and remaining allele of *CaESS1* in *C. albicans* indicating that at least one allele is required for growth under experimental conditions.

ESS1 plays an essential role in mitosis. Using recombinant DNA techniques and yeast genetics to engineer strains in which *ESS1* or *PIN1* gene expression is controlled by changing the carbon source, *ESS1* is shown to be required for cells to complete mitosis, a critical stage of the cell division cycle in which cells separate their chromosomes and organelles to form two complete cells (Lu et al., 1996). These results were confirmed using temperature-sensitive *ESS1* mutants. How exactly *ESS1* controls mitosis is not yet known, although work with the human homolog (*PIN1*) suggests it might bind and/or regulate mitotic phosphoproteins that are targets of the $p34^{cdc2}$ G2/M kinase (Shen et al. 1998). Work done with the S. cerevisiae ESS1 by the applicants and others suggest that the ESS1 protein is also important for transcription, perhaps by interacting with RNA polymerase II and other factors

required for transcription (Morris et al., 1999; Wu et al., 2000; Arevalo-Rodriguez et al., 2000).

Clues to how ESS1 family members might work: Some clues are provided by the discovery that ESS1 proteins contains two recognizable domains.

The first is a WW domain, identified in a number of seemingly unrelated proteins from different organisms, that has been shown to mediate protein-protein interactions important for intracellular signaling (Sudol 1996). Its is therefore likely, that ESS1 proteins contact other proteins via the WW domain.

The second domain is a peptidylprolyl cis-trans isomerase (PPIase) domain that is found in proteins that catalyze the isomerization of prolyl-containing peptides (Hemenway et al. 1993). PPIase proteins are thought to be important for protein folding, but are more widely known because they mediate the effects of immunosuppressive drugs like cyclosporin and FK506. The presence of a PPIase domain suggests that Ess1 may control the activity of other proteins by changing their conformational states by isomerization.

Isolation of the *Candida albicans* homolog of *ESS1*: To isolate a *Candida albicans* homolog of ESS1, Applicants isolated temperature sensitive mutations in the budding yeast *ESS1* gene. At the time of this invention, these were the only ts-conditional mutants available for an *ESS1* family member in any organism. A temperature sensitive mutant is also reported by Hani et al., "Mutations in a peptidylprolyl-cis/trans-isomerase gene lead to a defect in 3'-end formation of a pre-mRNA in *Saccharomyces cerevisiae*", J. Biol. Chem., Jan. 1999; 274(1):108-16. Yeast strains carrying these mutations ($ESS1^{ts}$) grow normally at the permissive temperature (30°C), but do not grow at the restrictive temperature (37°C). To clone the *Candida albicans ESS1* gene, a high-copy plasmid library containing *Candida*

albicans genomic DNA was transformed into one of the *ess1*^{ts} strains and colonies were selected for growth at the restrictive temperature. Among the colonies that grew, two carried an identical 8.5 kb DNA insert. Within this DNA insert, a gene was identified whose predicted protein product is 42% identical at the amino acid level to the Ess1 protein (Figure 1). Further complementation experiments showed that this gene is necessary and sufficient to rescue the lethal phenotype of an *ess1*^{ts} mutant. The gene was named *CaESS1* (*Candida albicans ESS1*). Of course, with the disclosure herein of the nucleic acid sequence for *CaESS1* as well as for primers for it, a preferred means for isolating *CaESS1* is by amplification (e.g., PCR) of the gene using the nucleic acid sequence for *CaESS1* or primers derived therefrom, such as primers disclosed herein.

CaESS1 as a Drug Target: The protein encoded by *CaESS1*, namely CaEss1, is an excellent target for antifungal drugs. The Ess1 protein is a highly conserved, essential PPIase whose activity is likely to be required for cell growth in a wide variety of pathogenic fungi. As mentioned previously, gene knockout experiments by the applicants suggest that CaESS1 is essential for growth in C. albicans, and work with other fungi, such as Aspergillus nidulans, shows that the ESS1 gene homolog is essential for growth in that organism (Dr. Anthony Means, Duke University, personal communication). In budding yeast, loss of Ess1 function causes mitotic arrest and nuclear fragmentation. This phenotype is not reversible, i.e. it is cytotoxic, not cytostatic. PPIases have been intensely studied as targets of immunosuppressive drugs but heretofore have not been fully explored as targets of antifungal drugs (see Hemenway et al. 1993; Georgopapadakou et al. 1994). Compounds such as rapamycin inhibit the growth of *Saccharomyces cerevisiae* by binding to a class of PPIases known as FKBPs. However, in yeast, none of the FKBP-class of PPIases nor

the cyclophilin-class of PPlases are essential for growth (Dolinski et al. 1997). Therefore, spontaneous mutations that abolish production of FKBP (or cyclophilin) remain viable, and are resistant to rapamycin (or cyclosporin A). This mechanism of resistance can pose a major clinical problem. In contrast, Ess1 protein is a parvulin-class PPlase (Rudd et al. 1995) and is absolutely required for growth in *Saccharomyces cerevisiae*; it is the only known PPlase that exhibits this property. Mutations that abolish production of Ess1 protein would be lethal; therefore, resistance by this mechanism would not occur. Known inhibitors of the FKBPs and cyclophilins (e.g. FK506, cyclosporin A) are not active against parvulin-class PPlases (Rudd et al. 1995). There are no specific inhibitors known to date. Finally, given the strong conservation of Ess1, it is likely that agents that inhibit *Candida albicans* Ess1 will also inhibit Ess1 homologs from other pathogenic yeasts such as *Cryptococcus neoformans* or *Aspergillus fumigatus*.

One possible concern is that anti-*CaESS1* or anti-CaEss1 drugs might also interfere with function of the human counterpart, *PIN1* or PIN1. However, the disclosure herein provides screening methods to overcome this possible concern. Furthermore, based on work in *Drosophila* (Maleszka et al.) and in mice (Fujimori et al., 1999) it is almost certain that PIN1 is not essential in humans, although there may be *PIN1*-related genes which have functional overlap with PIN1. Nonetheless, to avoid possible toxicity, Applicants screening methods aim to isolate compounds that preferentially target the fungal form of the enzyme over the human form. To do this *Saccharomyces cerevisiae* strains are engineered so they express, in a conditional manner both *Candida albicans CaESS1* and human *PIN1*. Using these strains, it is possible to identify compounds that preferentially inhibit the fungal form of the enzyme. In addition, by using variant screens, it is possible to identify compounds

that inhibit the human form of the enzyme, and these might usefully be developed as antiproliferative (anti-cancer) agents.

Thus, the present invention provides *CaESS1,* an essential *Candida albicans* gene, e.g., nucleic acid molecules therefor, and/or fragments or portions thereof, expression products therefrom, e.g., the protein CaEss1 or fragments or portions thereof, methods for making and using the gene, portions thereof and expression products therefrom, and to targeting the gene or portions thereof and/or the expression products therefrom for antifungal applications.

Inventive nucleic acid molecules include nucleic acid molecules having at least 70% identity or homology or similarity with *CaESS1* or probes or primers derived therefrom such as at least 75% identity or homology or similarity, preferably at least 80% identity or homology or similarity, more preferably at least 85% identity or homology or similarity such as at least 90% identity or homology or similarity, more preferably at least 95% identity or homology or similarity such as at least 97% identity or homology or similarity. The nucleotide sequence similarity or homology or identity can be determined using the "Align" program of Myers and Miller, ("Optimal Alignments in Linear Space", CABIOS *4*, 11-17, 1988) and available at NCBI. Using this method, the *CaESS1* gene is 53.7% identical to *S. cerevisiae ESS1* gene, and the *CaESS1* gene is 50.5% identical to human *ESS1* (*PIN1*) gene. Alternatively or additionally, the terms "similarity" or "identity" or "homology", for instance, with respect to a nucleotide sequence, is intended to indicate a quantitative measure of homology between two sequences. The percent sequence similarity can be calculated as $(N_{ref} - N_{dif})*100/N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will

have a sequence similarity of 75% with the sequence AATCAATC (N_{ref} = 8; N_{dif}=2). Alternatively or additionally, "similarity" with respect to sequences refers to the number of positions with identical nucleotides divided by the number of nucleotides in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman, 1983 PNAS USA 80:726), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics ™ Suite, Intelligenetics Inc. CA).. When RNA sequences are said to be similar, or have a degree of sequence identity with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

A probe or primer can be any stretch of at least 8, preferably at least 10, more preferably at least 12, 13, 14, or 15, such as at least 20, e.g., at least 23 or 25, for instance at least 27 or 30 nucleotides in *CaESS1* which are unique to *CaESS1* or which are in *CaESS1* and are least conserved among the *ESS1/PIN1* family. As to PCR or hybridization primers or probes and optimal lengths therefor, reference is also made to Kajimura et al., GATA 7(4):71-79 (1990).

RNA sequences within the scope of the invention are derived from the DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

Inventive amino acid molecules include amino acid molecules having at least 70% identity or homology or similarity with CaEss1 or portions thereof derived from the sequence provided herein such as at least 75% identity or homology or similarity, preferably at least 80% identity or homology or similarity, more preferably at least

85% identity or homology or similarity such as at least 90% identity or homology or similarity, more preferably at least 95% identity or homology or similarity such as at least 97% identity or homology or similarity. Amino acid sequence similarity or identity or homology can be determined using the BlastP program (Altschul *et al.*, Nucl. Acids Res. 25, 3389-3402) and available at NCBI. By this program, CaEss1 protein is 42% identical to *S. cerevisiae* Ess1 protein, and CaEss1 protein is 43% identical to human Ess1 (Pin1) protein. Alternatively or additionally, the terms "similarity" or "identity" or "homology", for instance, with respect to a nucleotide sequence, is intended to indicate a quantitative measure of homology between two sequences. The percent sequence similarity can be calculated as $(N_{ref} - N_{dif})*100/N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. The following references provide algorithms for comparing the relative identity of amino acid residues of two proteins : Needleman SB and Wunsch CD, "A general method applicable to the search for similarities in the amino acid sequences of two proteins," J. Mol. Biol. 48:444-453 (1970); Smith TF and Waterman MS, "Comparison of Bio-sequences," Advances in Applied Mathematics 2:482-489 (1981); Smith TF, Waterman MS and Sadler JR, "Statistical characterization of nucleic acid sequence functional domains," Nucleic Acids Res., 11:2205-2220 (1983); Feng DF and Dolittle RF, "Progressive sequence alignment as a prerequisite to correct phylogenetic trees," J. of Molec. Evol., 25:351-360 (1987); Higgins DG and Sharp PM, "Fast and sensitive multiple sequence alignment on a microcomputer," CABIOS, 5: 151-153 (1989); Thompson JD, Higgins DG and Gibson TJ, "ClusterW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, positions-specific gap penalties and weight matrix choice, Nucleic Acid Res., 22:4673-480

(1994); and, Devereux J, Haeberlie P and Smithies O, "A comprehensive set of sequence analysis program for the VAX," Nucl. Acids Res., 12: 387-395 (1984).

Like probes or primers, amino acids of the invention which are not full-length CaEss1 as depicted in Figure 1 can be any stretch of at least 8, preferably at least 10, more preferably at least 12, 13, 14, or 15, such as at least 20, e.g., at least 23 or 25, for instance at least 27 or 30 amino acids in CaEss1 which are unique to CaEss1 or which are in CaEss1 and are least conserved among the ESS1/PIN1 family. Alternatively or additionally, the amino acids of the invention which are not full length CaEss1 can be an epitopic region of CaEss1; for instance, to generate antibodies specific to CaEss1. One skilled in the art can determine an epitopic region of CaEss1 or an epitope of interest in CaEss1, without undue experimentation, from the disclosure herein and the knowledge in the art; see, e.g., WO 98/40500 regarding general information for determining an epitope of interest or an epitopic region of a protein.

The *CaESS1* gene or portions thereof can be expressed in yeast expression systems (see Examples or U.S. Patent No. 4,752,473), or other vectors. Methods for making and/or using such other vectors (or recombinants) for expression be by or analogous to the methods disclosed in: U.S. Patent Nos. 4,603,112, 4,769,330, 5,174,993, 5,505,941, 5,338,683, 5,494,807, 4,722,848, WO 94/16716, WO 96/39491, Paoletti, "Applications of pox virus vectors to vaccination: An update," PNAS USA 93:11349-11353, October 1996, Moss, "Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety," PNAS USA 93:11341-11348, October 1996, Smith et al., U.S. Patent No. 4,745,051 (recombinant baculovirus), Richardson, C.D. (Editor), Methods in Molecular Biology 39, "Baculovirus Expression Protocols" (1995 Humana Press Inc.), Smith et al., "Production of Huma Beta Interferon in Insect Cells Infected with a Baculovirus

Expression Vector," Molecular and Cellular Biology, Dec., 1983, Vol. 3, No. 12, p. 2156-2165; Pennock et al., "Strong and Regulated Expression of *Escherichia coli* B-Galactosidase in Infect Cells with a Baculovirus vector," Molecular and Cellular Biology Mar. 1984, Vol. 4, No. 3, p. 399-406; EPA 0 370 573, U.S. application Serial No. 920,197, filed October 16, 1986, EP Patent publication No. 265785, U.S. Patent No. 4,769,331 (recombinant herpesvirus), Roizman, "The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors," PNAS USA 93:11307-11312, October 1996, Andreansky et al., "The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors," PNAS USA 93:11313-11318, October 1996, Robertson et al. "Epstein-Barr virus vectors for gene delivery to B lymphocytes," PNAS USA 93:11334-11340, October 1996, Frolov et al., "Alphavirus-based expression vectors: Strategies and applications," PNAS USA 93:11371-11377, October 1996, Kitson et al., J. Virol. 65, 3068-3075, 1991; U.S. Patent Nos. 5,591,439, 5,552,143 (recombinant adenovirus), Grunhaus et al., 1992, "Adenovirus as cloning vectors," Seminars in Virology (Vol. 3) p. 237-52, 1993, Ballay et al. EMBO Journal, vol. 4, p. 3861-65, Graham, Tibtech 8, 85-87, April, 1990, Prevec et al., J. Gen Virol. 70, 429-434, PCT WO91/11525, Felgner et al. (1994), J. Biol. Chem. 269, 2550-2561, Science, 259:1745-49, 1993 and McClements et al., "Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes simplex virus-2 disease," PNAS USA 93:11414-11420, October 1996, and U.S. Patents Nos 5,591,639, 5,589,466, and 5,580,859 relating to DNA expression vectors, *inter alia*. See also WO 98/33510; Ju et al., Diabetologia, 41:736-739, 1998 (lentiviral expression system); Sanford et al., U.S. Patent No. 4,945,050; Fischbach et al. (Intracel), WO 90/01543; Robinson et al., seminars in

IMMUNOLOGY, vol. 9, pp.271-283 (1997) (DNA vector systems); Szoka et al., U.S. Patent No. 4,394,448 (method of inserting DNA into living cells); and McCormick et al., U.S. Patent No. 5,677,178 (use of cytopathic viruses), as well as other documents cited herein.

The expression product from the *CaESS1* gene or portions thereof can be useful for generating antibodies such as monoclonal or polyclonal antibodies which are useful for diagnostic purposes or to block CaEss1 enzyme activity.

Monoclonal antibodies are immunoglobulins produced by hybridoma cells. A monoclonal antibody reacts with a single antigenic determinant and provides greater specificity than a conventional, serum-derived antibody. Furthermore, screening a large number of monoclonal antibodies makes it possible to select an individual antibody with desired specificity, avidity and isotype. Hybridoma cell lines provide a constant, inexpensive source of chemically identical antibodies and preparations of such antibodies can be easily standardized. Methods for producing monoclonal antibodies are well known to those of ordinary skill in the art, e.g., Koprowski, H. et al., U.S. Pat. No. 4,196,265, issued Apr. 1, 1989, incorporated herein by reference.

Uses of monoclonal antibodies are known. One such use is in diagnostic methods, e.g., David, G. and Greene, H., U.S. Pat. No. 4,376,110, issued Mar. 8, 1983, incorporated herein by reference.

Monoclonal antibodies have also been used to recover materials by immunoadsorption chromatography, e.g. Milstein, C., 1980, Scientific American 243:66, 70, incorporated herein by reference.

Thus, products expressed from *CaESS1* or portions thereof are useful in immunoadsorption chromatography, as well as for generating antibodies for

diagnostic purposes. Furthermore, the expression products can be used in assays for detecting the presence of anti-CaEss1 antibodies.

For instance, the antibodies or expressed products can be used in assays analogous to those disclosed in U.S. Patents Nos. 5,591,645, 4,861,711, 5,861,319, 5,858,804, and 5,863,720, as well as in WO 86/04683, EP 154 749, WO 86/03839, and EP 186 799.

For instance, one can assay for *Candida albicans* by i) contacting, e.g., in a single test vessel, first and second capture agents respectively for first and second serological markers of *Candida albicans* infection, with a sample e.g., derived from a patient suspected to suffer therefrom (said sample suspected to contain said first and second markers, so as to permit any first and second disease markers in said sample to bind to said capture agents); ii) then contacting said capture agents with first and second labelled revealing agents bearing first and second labels respectively such that said first revealing agent gives a first signal corresponding to the amount of said first marker in said sample and said second revealing agent gives a second signal combinable with said first signal and corresponding to the amount of said second marker in said sample; iii) combining said first and second signals into a third signal, iv) detecting said third signal, and v) correlating the presence or absence of a *Candida albicans* infection in said patient with the strength of the third signal, wherein the strength of said first signal increases monotonically with increasing concentration of said first marker in said sample and the strength of said second signal decreases monotonically with increasing concentration of said second marker in said sample.

The sample can be blood. The first marker can be an antibody against CaEss1 or a fragment thereof expressed from *CaESS1* or a fragment thereof; or the first

capture agent could be CaEss1 or a fragment thereof, e.g., expressed from *CaESS1* or a fragment thereof.

The first revealing agent can be labeled CaEss1 or a fragment thereof, e.g., expressed from *CaESS1* or a fragment thereof. The second revealing agent can be a labelled antibody against CaEss1 or a fragment thereof, e.g., CaEss1 or a fragment thereof from expression of *CaESS1* or a fragment thereof. The first and second capture agents can be bound to solid supports, e.g., the same solid support, such as a well of a microtitre plate. The first and second revealing agents can bear the same label, e.g., a radioisotope such as ^{125}I, an enzyme such as. horseradish peroxidase, or fluorescent labels.

Thus, the nucleic acid molecules of the invention can be used to express inventive amino acids and amino acid molecules of the invention can be used in diagnostic applications involving antibody-binding, without undue experimentation, from the knowledge in the art and this disclosure.

The *CaESS1* DNA or inventive nucleic acid sequences can be used for diagnostic purposes. For instance, the nucleic acid sequences can be used to generate primers for diagnostic DNA, and the invention comprehends such primers. Primers are preferably derived from those parts of the *CaESS1* gene which are least conserved among the *ESS1/PIN1* family members. The gene or the primers can be used to detect if the gene is present in a sample or specimen and/or if the gene was expressed as RNA in a sample or specimen.

Accordingly, the inventive nucleotides can be used as probes to ascertain the presence of *Candida albicans* DNA in samples, as well as in the generation of PCR primers for replicating or cloning *Candida albicans* DNA. Methods for using DNA as probes or for preparing PCR primers are known in the art. In other words, the

CaESS1 gene or portions thereof are useful for generating primers for diagnostic PCR.

In diagnostic PCR, it is preferred that the primers bind specifically to *CaESS1*, i.e., specific hybridization is preferred. One way to ensure this is to select primers from the *CaESS1* gene sequence that are least conserved among *ESS1/PIN1* family members.

The term "specific hybridization" will be understood to mean that the nucleic acid probes of the invention are capable of stable, double-stranded hybridization to *Candida albicans*-derived DNA or RNA under conditions of high stringency, as the term "high stringency" would be understood by those with skill in the art (see, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Hames and Higgins, eds., 1985, Nucleic Acid Hybridization, IRL Press, Oxford, U.K.). Hybridization will be understood to be accomplished using well-established techniques, including but not limited to Southern blot hybridization, Northern blot hybridization, *in situ* hybridization and, most preferably, Southern hybridization to PCR-amplified DNA fragments.

The nucleic acid hybridization probe of the invention may be obtained by use of the polymerase chain reaction (PCR) procedure, using appropriate pairs of PCR oligonucleotide primers as provided herein or derived from the *CaESS1* gene sequence provided herein. See U.S. Pat. Nos. 4,683,195 to Mullis et al. and 4,683,202 to Mullis.

The invention provides oligonucleotides for *in vitro* amplification using any of a variety of amplification protocols known in the art. Preferably, the invention

provides oligonucleotides for performing polymerase chain reaction (PCR). See U.S. Pat. Nos. 4,683,195 to Mullis et al. and 4,683,202 to Mullis.

The invention will thus be understood to provide oligonucleotides, specifically, pairs of oligonucleotides, for use as primers for the *in vitro* amplification of *Candida albicans* DNA samples and fragments thereof. In the practice of this invention, the pairs of oligonucleotides herein provided will be understood to comprise two oligonucleotides, comprising from about 8 to about 30 nucleotide residues apiece, said oligonucleotides specifically hybridizing to sequences flanking a nucleic acid to be amplified, wherein the oligonucleotides hybridize to different and opposite strands of the DNA target. The oligonucleotides of the invention are preferably derived from the nucleic acid primers discussed below or from the *CaESS1* gene disclosed herein. As used in the practice of this invention, the term "derived from" is intended to encompass the development of such oligonucleotides from the nucleic acid sequence of the *CaESS1* gene or the primers herein disclosed, from which a multiplicity of alternative and variant oligonucleotides can be prepared. In particular, the invention provides oligonucleotides having a sequence that is substantially complementary to the corresponding sequence of the nucleic acid hybridization probe. As used herein, the term "substantially corresponding to" is intended to encompass oligonucleotides comprising sequence additions, deletions and mismatches, wherein certain nucleotide residues of the oligonucleotide sequence are not optimally complementary (e.g., A-C or G-T) or are non-complementary (e.g., A-G or T-C) to the corresponding sequence of the nucleic acid hybridization probe, provided that such oligonucleotides retain the capacity to specifically amplify *CaESS1*.

Nucleic acids, e.g., *CaESS1*, and oligonucleotides therefrom, such as primers disclosed herein and derivable from the *CaESS1* sequence of the present invention (e.g., portions of the disclosed *CaESS1* which are about 8 to 30 or more nucleotides in length and bind with sufficient specificity to *CaESS1* are useful as diagnostic tools for detecting the existence of a *Candida albicans* infection or the presence of *Candida albicans*. Such diagnostic reagents comprise nucleic acid hybridization probes of the invention and encompass paired oligonucleotide PCR primers, as described above.

Methods provided by the invention include blot hybridization, *in situ* hybridization and *in vitro* amplification techniques for detecting the presence of *Candida albicans* in a sample such as a biological sample. Appropriate biological samples advantageously screened using the methods described herein include blood, serum, saliva and other body fluids, and other potential sources of infection.

In the detection methods of the invention, production of a specific DNA fragment produced by *in vitro* amplification of a template DNA sample is detected by agarose gel electrophoresis, ethidium bromide staining and ultraviolet transillumination of ethidium bromide stained gels, performed using conventional techniques (Sambrook et al., *supra*), or detection by sequence detection systems using fluorogenic or other labeled probes that rely on automatic or automated detection instrumentation. In instances where a greater degree of specificity is required, hybridization of such agarose gels probed with a detectably-labeled nucleic acid hybridization probe of the invention is performed using standard techniques (Sambrook et al., *supra*). In each of these embodiments of the methods of the invention, a sufficient amount of a specific PCR-amplified DNA fragment is produced to be readily detected. For the purposes of this invention, the term "a sufficient amount of a specific PCR-amplified DNA fragment" is defined as that amount

required to be detected, either by visualization of ethidium bromide-stained agarose gels or autoradiographic or other development of a blot hybridized with a detectably-labeled probe.

It will be understood that a sufficient quantity of a specific PCR amplified DNA fragment is prepared in PCR amplification reactions by performing a number of cycles required to produce said sufficient amount of the specific DNA fragment. The number of cycles in each PCR required to produce said sufficient amount of a specific DNA fragment will be understood to depend on the oligonucleotide primers, buffers, salts and other reaction components, the amount of template DNA and the PCR cycling times and temperatures. It will also be understood that the optimization of these parameters are within the skill of the worker of ordinary skill to achieve with no more than routine experimentation.

Detectably-labeled probes as provided by the invention are labeled with biotin, a radioisotope (including ^{3}H, ^{14}C, ^{35}S and ^{32}P), a fluorescent label (including fluoresceinisothiocyanate), and an antigenic label. The detectable label is incorporated into the probe during synthetic preparation of the probe, whereby the probe is alternatively end-labeled or labeled by the incorporation of labeled nucleotides into the synthesized probe.

The invention also provides a PCR-based method for preparing a nucleic acid hybridization probe of the invention. In these embodiments, template DNA comprises a recombinant genetic construct of the invention. A detectably-labeled nucleic acid hybridization probe is prepared by performing PCR amplification using a pair of oligonucleotide primers specific for sequences flanking the position of the nucleic acid insert. Detectable label is incorporated into the nucleic acid hybridization probe

by direct end-labeling of PCR primers or incorporation of detectably-labeled nucleotide triphosphates into the probe nucleic acid.

PCR comprising the methods of the invention is performed in a reaction mixture comprising an amount, typically between <10 ng-200 ng template nucleic acid; 50-100 pmoles each oligonucleotide primer; 1-1.25 mM each deoxynucleotide triphosphate; a buffer solution appropriate for the polymerase used to catalyze the amplification reaction; and 0.5-2 Units of a polymerase, most preferably a thermostable polymerase (e.g., Taq polymerase or Tth polymerase).

The invention thus provides diagnostic assays for the specific detection of *Candida albicans*. These diagnostic assays include nucleic acid hybridization assays, using the nucleic acids of the invention or specifically-hybridizing fragments thereof, for sensitive detection of fungal genomic DNA and/or RNA. Such assays include various blot assays, such as Southern blots, Northern blots, dot blots, slot blots and the like, as well as *in vitro* amplification assays, such as the polymerase chain reaction assay (PCR), reverse transcription-polymerase chain reaction assay (RT-PCR), ligase chain reaction assay (LCR), and others known to those skilled in the art. Specific restriction endonuclease digestion of diagnostic fragments detected using any of the methods of the invention, analogous to restriction fragment linked polymorphism assays (RFLP) are also within the scope of this invention.

Accordingly, the invention relates to compositions and methods for detecting and/or diagnosing *Candida albicans*.

Moreover, the identification of the *CaESS1* gene allows for identifying compounds or agents that specifically bind to and/or inhibit the gene, or portions thereof and/or expression products therefrom, and methods for preventing and/or treating *Candida albicans* and/or symptoms or conditions associated therewith, as

well as generally for making and using such compounds or agents. Thus, the invention relates to antifungal preparations and/or compositions and methods for making and using them.

As discussed herein, the *CaESS1* gene and portions thereof are useful for generating or expressing the CaEss1 protein and epitopic portions thereof (epitopic portions of the protein can be derived from, generated by, or expressed from those parts of the *CaESS1* gene which are least conserved among the *ESS1/PIN1* family members). The protein or portions thereof is useful for generating antibodies, such as monoclonal and/or polyclonal antibodies. In addition to using these antibodies for diagnostic purposes, the antibodies can be used to block CaEss1 activity.

Additionally or alternatively, a CaEss1 inhibitor can be a compound which selectively inhibits growth of *S. cerevisiae* not containing an endogenous *ESS1* gene but rather *CaESS1* and uninduced *PIN1* (e.g., on a glucose medium) (see Figs. 2, 3) and preferably does not inhibit or significantly inhibit PIN1, e.g., does not inhibit or significantly inhibit *S. cerevisiae* not containing an endogenous *ESS1* gene but rather an induced *PIN1* (e.g., on a glucose/galactose medium) (see Fig. 4).

Compositions which indeed inhibit *PIN1* are nonetheless useful as antiproliferatives, e.g., antineoplastics, anti-tumor agents or anti-cancer agents.

Compositions containing inhibitors of CaEss1 or containing antiproliferatives are within the scope of the invention. Compositions for use in the invention can be prepared in accordance with standard techniques well known to those skilled in the pharmaceutical or medical arts. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration. The compositions can be administered alone,

or can be co-administered or sequentially administered with other compositions of the invention or with other prophylactic or therapeutic compositions.

Examples of compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, genital (e.g., vaginal), etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular, intravenous, intraarterial, intralymphatic, or intraperitoneal administration (e.g., injectable administration) such as sterile suspensions or emulsions. In such compositions the active agent can be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like.

The compositions of the invention may be packaged in a single dosage form for injection administration or orifice administration. Accordingly, compositions in forms for such administration routes are envisioned by the invention. And again, the effective dosage and route of administration are determined by known factors, such as age, sex, weight, condition and nature of patient, as well as LD_{50} and other screening procedures which are known and do not require undue experimentation. Dosages of each active agent can range from a few to a few hundred micrograms, e.g., 5 to 500 μg.

The compositions can be administered in intervals without undue experimentation by the skilled artisan considering the disclosure herein and the knowledge in the art and known factors such as age, sex, weight, condition, and nature of the patient as well as LD_{50} and other screening procedure results; for instance, compositions can be administered in a regimen to or serially akin to administration protocols for known antifungals or antiproliferatives; or preferably over a shorter duration or in lesser doses than known antifungals or antiproliferatives.

Accordingly, the invention relates to diagnostic compositions and methods, as well as therapeutic or preventive compositions and methods.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

EXAMPLE 1: CLONING *CaESS1*

The *Candida albicans* homolog of *ESS1* was cloned by functional complementation of a temperature-sensitive *Saccharomyces cerevisiae* strain, *ess1*-L94P^{ts}. This strain was created by Applicants by methods described in Adachi et al., *Nucl. Acids Res.* 22:4229-4233, 1994, and is similar to temperature sensitive mutants of Hani et al., *J Biol Chem* 1999 Jan 1; 274(1):108-16. Applicants screened a *Candida albicans* genomic DNA library to search for sequences capable of complementing the no-growth phenotype of the *ess1*-L94P^{ts} strain under non-permissive conditions. The details are as follows.

A *Candida albicans* genomic DNA library in YEp352 was obtained from Navarro-Garcia (Mol. Cell Biol., *15*, 2197-2206). The library carried 5-10 kb-sized genomic DNA fragments generated by *Sau3A* partial digestion of the *Candida albicans* 1001 genome and contained 2 x 10^5 independent members. The library was amplified in *Escherichia coli* 20-fold over the original size and purified prior to use.

The *ess1*-L94P^{ts} yeast cells were transformed with a total of 200 µg of the amplified *Candida albicans* genomic library using a standard lithium acetate protocol (Ito *et al.*, J. Bact. *53*, 163-168). Transformation was done in 33 independent aliquots of 150 ul cells each using approximately 6 ug of library DNA. Based on the transformation efficiency (8300 transformants/µg), approximately 2 x 10^6 transformants (10-fold over library size) were expected. The 33 transformations were

pooled in groups of three (11 pools; 1.5 x 10^5 transformants per pool) and grown overnight at 30°C in 4 ml of CSM-minus-ura liquid medium. The cells were concentrated by centrifugation and plated onto CSM-minus-ura plates and incubated at 37°C for 3 days. 150 colonies (i.e. positives) were obtained. The positives were serially passaged three times by streaking for single colonies and incubating at 37°C for 3 days. Colonies representing 26 of the original 150 postives remained viable under these conditions.

To determine whether the complementing activity was plasmid-linked, these 26 postives were streaked onto 5-flouroorotic acid (5-FOA) containing plates at 37°C. 5-FOA selects against the *URA3* gene carried on the library plasmids. Therefore, those positives that did not grow on 5-FOA plates carried complementing activity that was plasmid-linked. A total of 5 out of the 26 positives did not grow on 5-FOA plates at 37°C; these were the desired clones.

Plasmids were rescued out of all 26 positives and retransformed into *ess1*-L94P^{ts}. Of these, only those 5 which had previously been shown to not grow on 5-FOA at 37°C were able to rescue the no-growth phenotype of *ess1*-L94P^{ts} at 37°C.

Restriction mapping of the 5 complementing clones revealed that they belonged to 2 different groups. Two clones comprising one group contained an identical ~8.5 Kb insert. DNA sequence analysis revealed the existence of an *ESS1* homolog in *Candida albicans*. The amino acid identity to *Saccharomyces cerevisiae ESS1* is 42%. The other group carried an unrelated *Candida albicans* high copy suppressor.

Figure 1 shows the nucleotide sequence and predicted expression product from *CaESS1*. From this disclosure, a preferred method for cloning *CaESS1* is by using

PCR amplification employing primers derived from *CaESS1* such as primers disclosed herein.

EXAMPLE 2: CaEss1 INHIBITORS

On the basis of Applicants' discovery of the *CaESS1* gene and the fact that it is functionally equivalent to the *Saccharomyces cerevisiae ESS1* gene, strains of *Saccharomyces cerevisiae* can be engineered for use in screens to identify chemical inhibitors of *CaESS1* function. Such compounds can be used as antifungal drugs. Such compounds are identified based on their ability to block cell growth in *Saccharomyces cerevisiae* as a result of interference with *CaESS1* function. This interference might or might not be due to direct inhibition of PPIase enzyme activity.

In addition, prior work demonstrated that the human *ESS1* homolog (*PIN1*) is also a functionally equivalent to the *Saccharomyces cerevisiae ESS1* gene (Lu *et al.*, Nature *380*, 544-547). Therefore, *Saccharomyces cerevisiae* strains can be engineered to conditionally express the products of both *CaESS1* or human *PIN1* in the same cells. Such strains can be used to identify inhibitor compounds that selectively inhibit *CaESS1* function, but not human *PIN1* function (or that inhibit *PIN1* to a lesser degree). Finally, *Saccharomyces cerevisiae* strains expressing human *PIN1* can be used in screens to identify compounds that inhibit human *PIN1* function. These compounds can be antiproliferative (*e.g.* anticancer) drugs, and these compounds may also inhibit *CaESS1* function.

A representative example of the general design of such strains and how the inhibitor screens work are shown in Figures 2, 3, and 4. Note: The strategies outlined here are easily adaptable to screen for inhibitors of potential *ESS1* homologs from other pathogenic fungi, *e.g. Cryptococcus neoformans* or *Aspergillus fumigatus.*

Figure 2 shows the strategy for identifying inhibitors specific for *Candida albicans*. The yeast *Saccharomyces cerevisiae* is engineered to express both the *Candida albicans CaESS1* gene and the human *PIN1* gene. The endogenous *Saccharomyces cerevisiae ESS1* gene is deleted. The *hPIN1* gene is expressed from an inducible promoter (e.g., the *GAL1* promoter) so that it can be turned off and on by changes in the culture medium (strains can also bear other changes such as mutations that favor higher efficiency drug entry or retention and the *CaESS1* gene might be modified so as to make it more sensitive to the effects of chemical inhibitors, for example, by the introduction of conditional-lethal mutations, as the use of such mutations, e.g., temperature-sensitive mutations, for screens carried out under permissive or semi-permissive conditions would sensitize cells to the effects of *CaESS1* or CaEss1 inhibitors).

Figure 3 shows a screen for *CaESS1* or CaEss1 inhibitors. Cultures of specially engineered *Saccharomyces cerevisiae* (see Fig. 2) are grown in duplicate plates under different conditions, e.g., one condition is glucose, another condition is galactose (or a mixture of glucose/galactose which produces low but sufficient levels of *hPIN1* expression for cell viability, and this might be useful because massive overproduction of *hPIN1*, e.g., in galactose, might overcome compounds that inhibit both *CaESS1* or CaEss1 and *hPIN1* or PIN1, thus leading to possible false positives, i.e., possible compounds that inhibit both the fungal and human gene function). Cells grown in glucose-containing medium express *CaESS1* but not *hPIN1*; cells grown in galactose-containing medium express both *CaESS1* and *hPIN1*; potential inhibitory compounds are applied to each well in duplicate, yeast growth is monitored. Many compounds may have no effect. Compounds in which yeast cell growth in both glucose and galactose plates (B2 and D3 in Fig. 3) inhibit both *CaESS1* or CaEss1 and

hPIN1 or hPIN1 (non-specific inhibitors of yeast cell growth); and, compounds which inhibit only yeast growth in glucose plate (C5 in Fig. 3) are *CaESS1* or CaEss1-specific inhibitors.

Figure 4 shows a screen for *hPIN1* or hPIN1 inhibitors. Cultures of specially engineered *Saccharomyces cerevisiae* in which the *ESS1* gene is deleted and a *hPIN1* under the control of a promoter such as *GAL1* is present are grown in duplicate plates under different conditions, e.g., one condition is a mixture of glucose and galactose, another condition is galactose; cells grown in medium containing a mixture of glucose/galactose express low levels of *hPIN1*; cells grown in galactose-containing medium express high levels of *hPIN1*; potential inhibitory compounds are applied to each well in duplicate, yeast growth is monitored. Many compounds may have no effect. Compounds in which yeast cell growth in both glucose/galactose and galactose plates (B2 and D3 in Fig. 4) non-specifically inhibit yeast cell growth; and compounds which inhibit only yeast growth in glucose/galactose plate (C5 in Fig. 4) are *hPIN1* or hPIN1 inhibitors, as massive overproduction of *hPIN1* (in galactose) overcomes the inhibitory effect by titrating out the inhibitor.

EXAMPLE 3: *CaESS1* AMPLIFICATION; PRIMERS THEREFOR

The DNA oligonucleotide primers, OW-216 AND OW-221, are based on the *CaESS1* sequence, and preferentially amplify *CaESS1* in a diagnostic PCR reaction. The reaction product is a 453 bp of DNA that corresponds to a portion of the *CaESS1* open reading frame. These primers will not amplify human *PIN1*, or *Saccharomyces cerevisiae ESS1* sequences (the corresponding sequences from *PIN1* and *ESS1* are shown below the *CaESS1* primers; they are clearly different).

OW-216 5' CCA-GAT-GGT-ATA-AGT-AGA-AC-3' (*C. albicans CaESS1*) (SEQ ID NO:3)

5' ATC-AAC-GGC-TAC-ATC-CAG-AA-3' (human *PIN1*) (SEQ ID NO: 4)

5' GAC-GCT-ACG-GAC-GAA-CTG-AA-3' (*S. cerevisiae ESS1*) (SEQ ID NO: 5)

OW-221 5' CAA-TGA-CGG-GAA-ACG-TTC-CG-3' (*C. albicans CaESS1*) (SEQ ID NO: 6)

5' GGG-AGT-GGG-GAC-CCC-AGG-GC-3' (human *PIN1*) (SEQ ID NO: 7)

5' GTC-ATC-TGG-AGA-GGA-AAA-GA-3' (*S. cerevisiae ESS1*) (SEQ ID NO:8)

Table 1. Results of gene knockout of *CaESS1* in *Candida albicans*.

Transformation of *caess1/CaESS1**	# Transformants analyzed	Method of analysis	Locus of insertion
CaGD-1	11	Southern + PCR	1st allele
"	19	PCR	1st allele
CaGD-2	11	Southern + PCR	1st allele
"	19	PCR	1st allele
total	**60**		**60/60 in 1st allele**

* CaGD-1 and CaGD-2 represent independently-derived 5-FOA revertants from different CAI4-disrupted strains (*hisG-CaURA3-hisGΔCaESS1*).

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope thereof.

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WHAT IS CLAIMED IS:

1. An isolated or purified nucleic acid molecule comprising a nucleotide sequence encoding CaEss1, or having at least 70% homology thereto.

2. The isolated or purified nucleic acid molecule of claim 1 comprising the nucleotide sequence set forth in Figure 1 (SEQ ID NO:1), or at least 70% homology thereto.

3. An isolated or purified polypeptide comprising an amino acid sequence having the enzymatic activity of CaEss1, or at least 70% homology thereto.

4. The isolated or purified polypeptide of claim 3 comprising the amino acid sequence set forth in Figure 1 (SEQ ID NO:2).

5. A primer or probe which specifically hybridizes to the nucleic acid molecule of claim 1 or 2.

6. The primer or probe of claim 5 comprising OW-216 or OW-221 (SEQ ID NOS: 3, 6).

7. A method for detecting *Candida albicans* in a sample comprising detecting the presence therein of a nucleic acid molecule of claim 1 or 2.

8. A method for detecting *Candida albicans* in a sample comprising detecting the presence therein of a polypeptide of claim 3 or 4 or of an antibody which binds to such a polypeptide.

9. An antibody which binds to the polypeptide of claim 3 or 4.

10. A diagnostic composition comprising the polypeptide of claim 9.

11. A diagnostic composition comprising the nucleic acid molecule of claim 1 or 2.

12. A diagnostic composition comprising the primer or probe of claim 5.

13. A diagnostic composition comprising the primer or probe of claim 6.

14. A compound which inhibits *Candida albicans* by inhibiting CaEss1 or *CaESS1*.

15. The compound of claim 14 comprising an antibody which binds to CaEss1.

16. The compound of claim 14 which selectively inhibits growth of yeast transformed to contain and express *CaESS1* and/or *PIN1* and not an endogenous *ESS1*, when *CaESS1* is expressed but not when *PIN1* is expressed.

17. An antiproliferative compound which selectively inhibits growth of yeast transformed to contain and express *PIN1* and not an endogenous *ESS1*, and this inhibition can be overcome by high levels of *PIN1* expression.

18. A method for preventing or treating *Candida albicans* comprising administering a compound as claimed in any of claims 14, 15 or 16.

19. A method for preventing human cell growth comprising administering a compound as claimed in claim 17.

20. A vector comprising the nucleic acid molecule of claim 1 or 2.

21. A method for preparing CaEss1 comprising transforming a vector to contain the isolated nucleic acid molecule of claim 1 or 2 and obtaining expression thereof.

22. The method of claim 21 wherein the vector is a yeast.

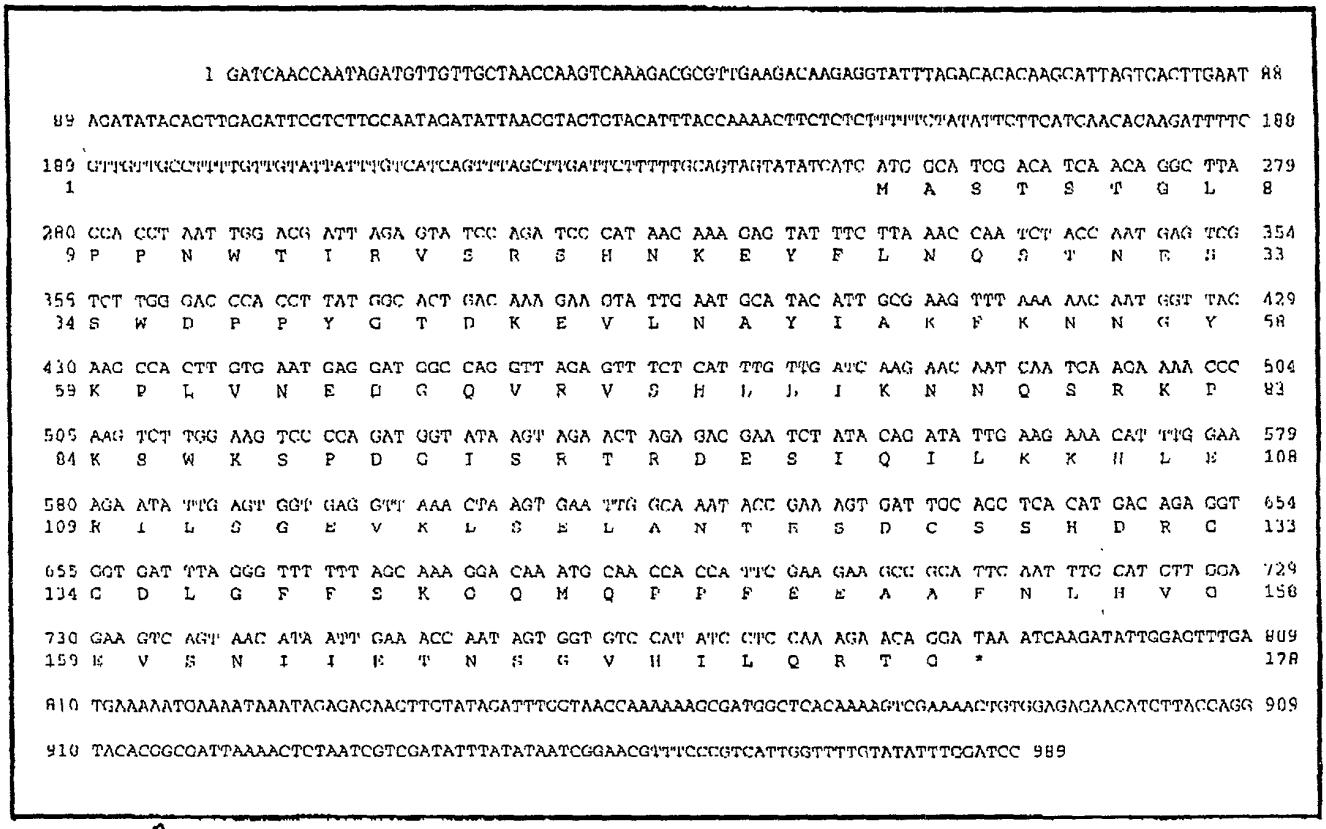
23. A method for obtaining an isolated nucleic acid molecule encoding CaEss1 comprising performing a polymerase chain reaction on a

sample suspected to contain *CaESS1* using primers or probes which specifically hybridize thereto.

ABSTRACT OF THE DISCLOSURE

Disclosed and claimed is the *CaESS1* gene, portions thereof such as primers or probes, expression products therefrom, and methods for using the gene, and expression products; for instance, for diagnostic, therapeutic or preventive compositions.

Figure 1A. The *CaESS1* gene of *Candida albicans*.



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                1 GATCAACCAATAGATGTTGTTGCTAACCAAGTCAAAGACGCGTTGAAGACAAGAGGTATTTAGACACACAAGCATTAGTCACTTGAAT 88

               89 AGATATACAGTTGAGATTCGTCTTCCAATAGATATTAACGTAGTCTACATTTACCAAAACTTCTCTCTTTTTCTTATATTCTTCATCAACACAAGATTTTC 188

              189 GTTGGTTGCCTTTTTGTTGTATTATTTGTCATCAGTTTAGCTTGATTCTTTTTGCAGTAGTATATCATC ATG GCA TCG ACA TCA ACA GGC TTA  279
                1                                                                     M   A   S   T   S   T   G   L    8

              280 CCA CCT AAT TGG ACG ATT AGA GTA TCC AGA TCC CAT AAC AAA GAG TAT TTC TTA AAC CAA TCT ACC AAT GAG TCG  354
                9 P   P   N   W   T   I   R   V   S   R   S   H   N   K   E   Y   F   L   N   Q   S   T   N   E   S   33

              355 TCT TGG GAC CCA CCT TAT GGC ACT GAC AAA GAA GTA TTG AAT GCA TAC ATT GCG AAG TTT AAA AAC AAT GGT TAC  429
               34 S   W   D   P   P   Y   G   T   D   K   E   V   L   N   A   Y   I   A   K   F   K   N   N   G   Y   58

              430 AAC CCA CTT GTG AAT GAG GAT GGC CAG GTT AGA GTT TCT CAT TTG TTG ATC AAG AAC AAT CAA TCA AGA AAA CCC  504
               59 K   P   L   V   N   E   D   G   Q   V   R   V   S   H   L   L   I   K   N   N   Q   S   R   K   P   83

              505 AAG TCT TGG AAG TCC CCA GAT GGT ATA AGT AGA ACT AGA GAC GAA TCT ATA CAG ATA TTG AAG AAA CAT TTG GAA  579
               84 K   S   W   K   S   P   D   G   I   S   R   T   R   D   E   S   I   Q   I   L   K   K   H   L   E   108

              580 AGA ATA TTG AGT GGT GAG GTT AAA CTA AGT GAA TTG GCA AAT ACC GAA AGT GAT TGC AGC TCA CAT GAC AGA GGT  654
              109 R   I   L   S   G   E   V   K   L   S   E   L   A   N   T   E   S   D   C   S   S   H   D   R   G   133

              655 GGT GAT TTA GGG TTT TTT AGC AAA GGA CAA ATG CAA CCA CCA TTC GAA GAA GCC GCA TTC AAT TTG CAT CTT GGA  729
              134 G   D   L   G   F   F   S   K   G   Q   M   Q   P   P   F   E   E   A   A   F   N   L   H   V   G   158

              730 GAA GTC AGT AAC ATA ATT GAA ACC AAT AGT GGT GTC CAT ATC CTC CAA AGA ACA GGA TAA ATCAAGATATTGGAGTTTGA 809
              159 E   V   S   N   I   I   E   T   N   S   G   V   H   I   L   Q   R   T   G   *                       178

              810 TGAAAAATGAAAATAAATAGAGACAAGTTCTATAGATTTCGTAACCAAAAAAGCGATGGCTCACAAAAGTCGAAAACTGTGGAGACAACATCTTACCAGG 909

              910 TACACGGCGATTAAAACTCTAATCGTCGATATTTATATAATCGGAACGTTTCCCGTCATTGGTTTTGTATATTTCGATCC 989
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Figure 1A. Complete nucleotide sequence of the *CaESS1* gene from *Candida albicans* and its predicted translation product. The *CaESS1*-encoded protein is 177 amino acids long and has a predicted MW of 19.6 Kd. It is 42% identical to the *ESS1* protein of *Saccharomyces cerevisiae*.

Figure 1B. Gene Knockout of *CaESS1* in *Candida albicans*.

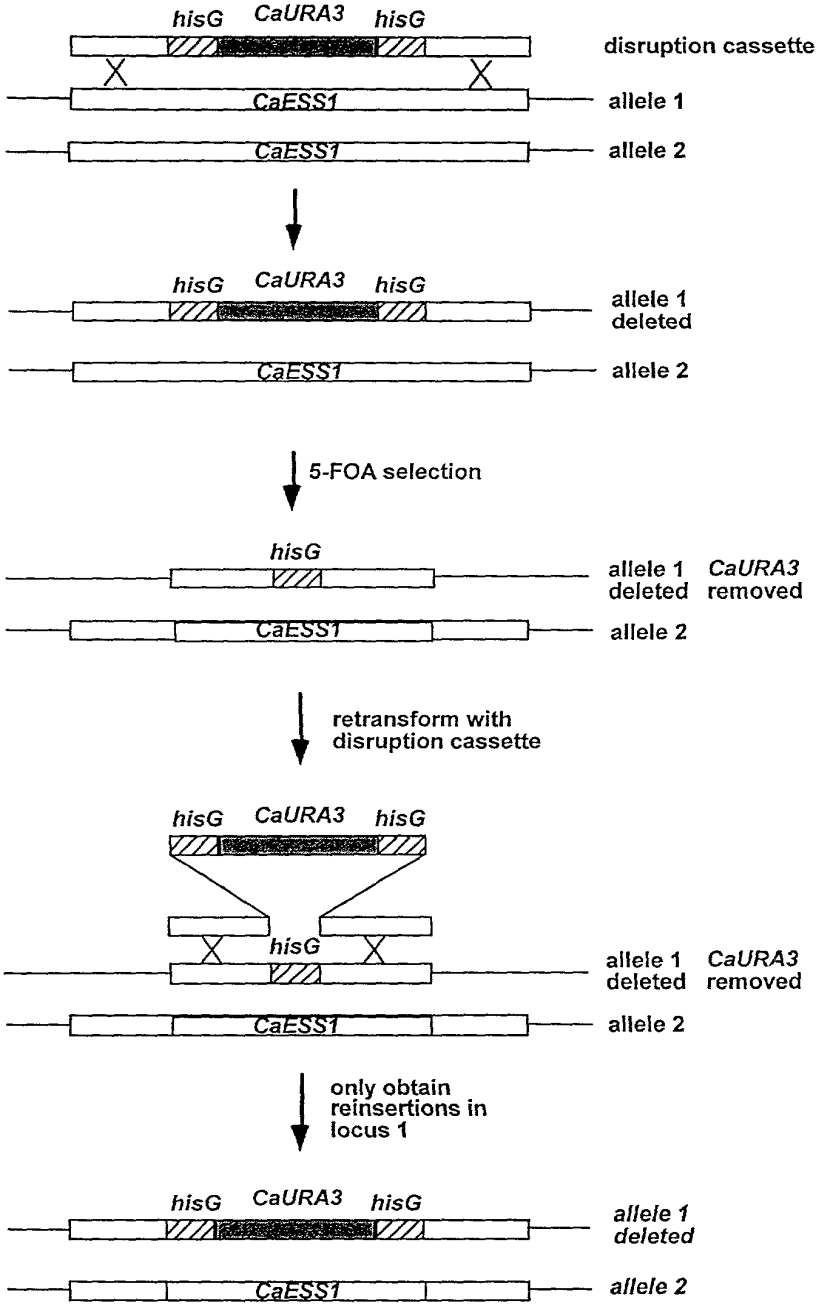


Figure 1B. *CaEss1* was deleted in strain CAI4 by the method of Fonzi and Irwin (1993). Ura+ transformants were selected, genomic DNA was prepared and analyzed by Southern hybridization and by PCR. Results confirmed homologous recombination and gene deletion of the first allele as outlined in the figure. The *CaURA3* gene was then removed by selection with 5-FOA, and diploid disruption strains (*caess1/CaESS1*) were used for retransformation with the *hisG-CaURA3-hisGΔCaESS1* disruption cassette as before. No homozygous deletion strains (*caess1/caess1*) were obtained (see Table 1). Instead the *hisG-CaURA3-hisGΔCaESS1* cassette reinserted into the already disrupted allele in all Ura+ transformants analyzed.

Figure 2. Yeast Strain to Identify Inhibitors Specific for *Candida albicans* CaESS1

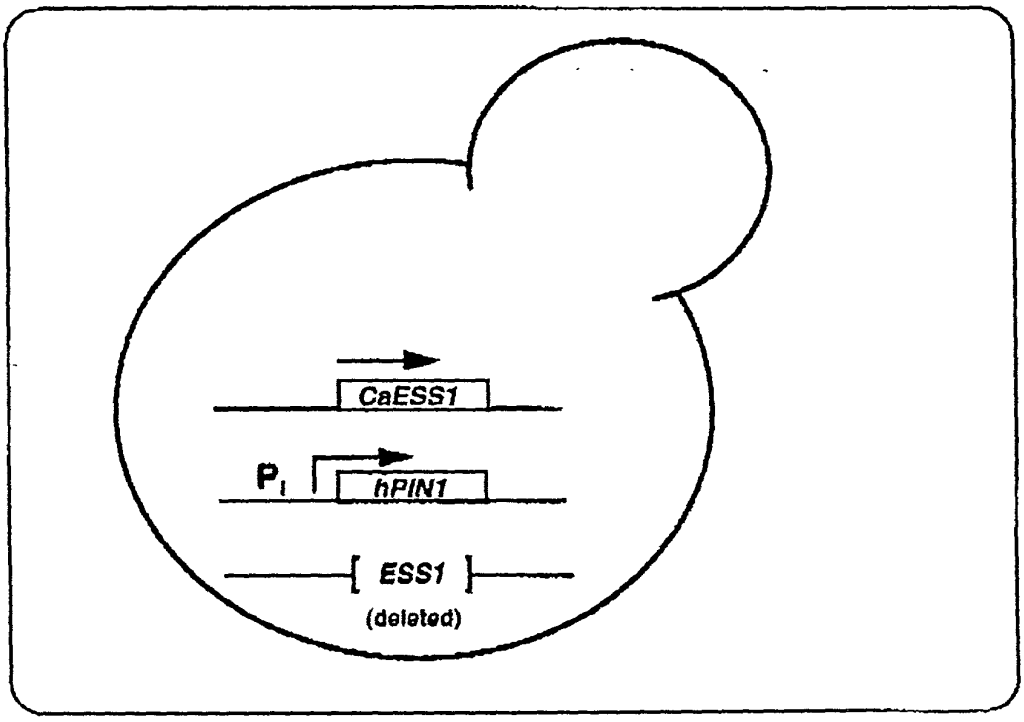


Figure 3. Screen for *CaESS1* Inhibitors

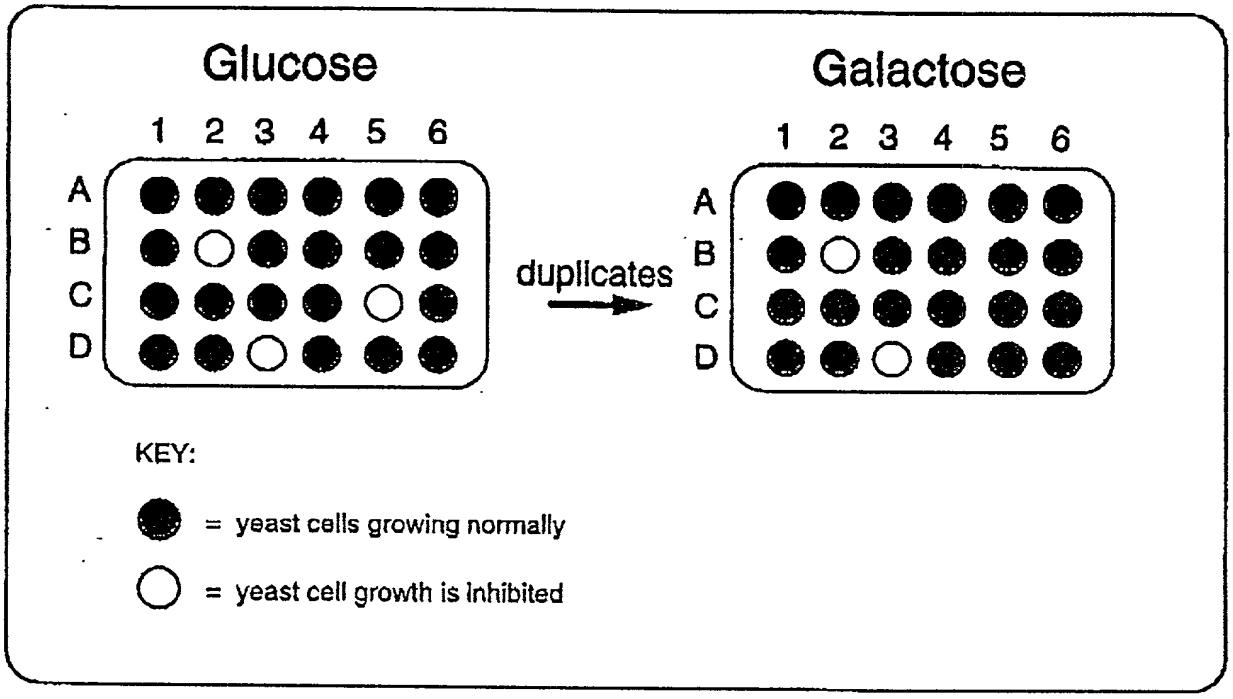
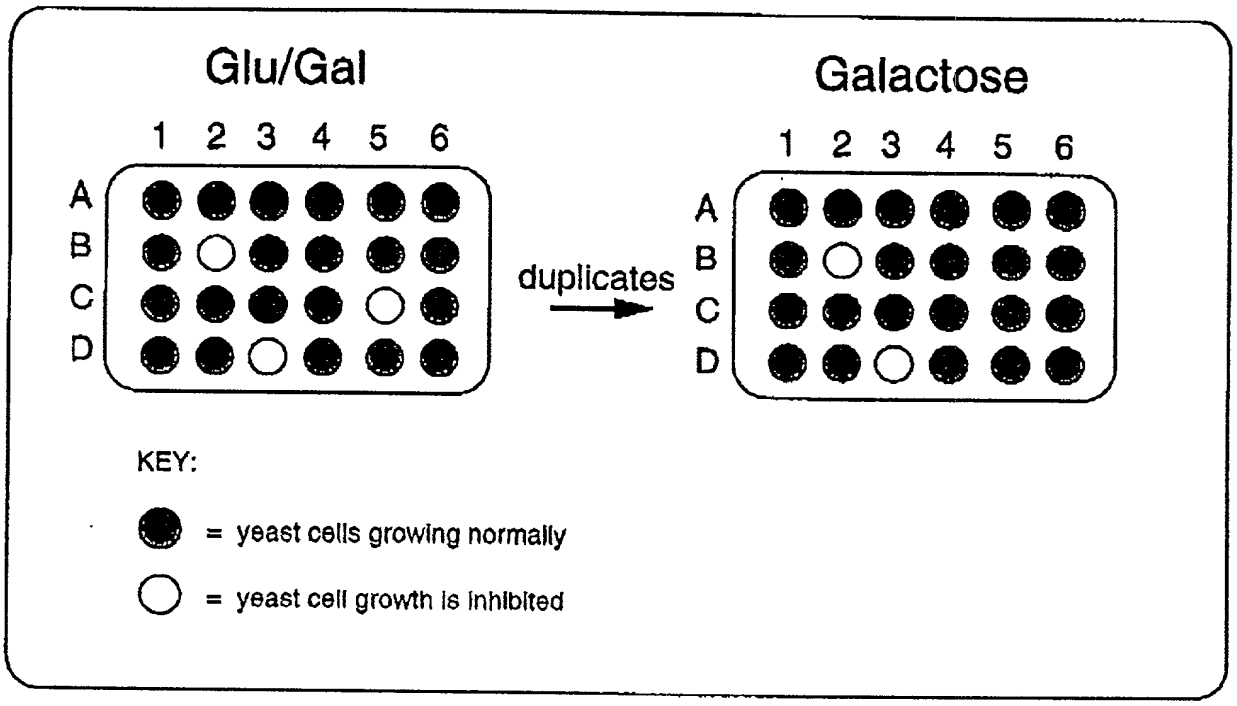


Figure 4. Screen for *hPIN1* Inhibitors



FLH Docket No
454311-2200.1

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes reference to PCT International Applications)

FROMMER LAWRENCE & HAUG, LLP
File No.: 454311-2200.1

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural, names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED:

***CaESSI*: A CANDIDA ALBICANS GENE, METHODS FOR MAKING AND USING, AND TARGETING IT AND ITS EXPRESSION PRODUCTS FOR ANTIFUNGAL APPLICATIONS**

the specification of which:

__ is attached hereto

X was filed with/transmitted to USPTO on February 18, 2000 as:

__ United States Application Serial No._____

__ as the National Phase or Continuation or Continuation-in-Part of PCT Application No. _____, filed _____, designating the U.S., and published as_____ on _____,

__X__ with amendments through DATE EVEN HEREWITH (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a) - (d) or § 365 (b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

60/121,246 (Application Number) FEBRUARY 23, 1999 (Filing Date)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or § 365 (c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior United States or PCT International application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.:	Filed (Day/Month/Year)	PCT Application No.	Status (patented, pending, abandoned)

I hereby appoint Thomas J. Kowalski, Registration No. 32,147, and FROMMER LAWRENCE & HAUG, LLP or their duly appointed associates, my attorneys or agents, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and to insert the Serial Number of the application in the space provided above, and specify that all communications about the application are to be directed to the following correspondence address:

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to the attention of:
Thomas J. Kowalski

I hereby declare that all statements made herein of my own knowledge are true and that

all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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NOTE: In order to qualify for reduced fees available to Small Entities, each inventor and any other individual or entity having rights to the invention must also sign an appropriate separate "Verified Statement (Declaration) Claiming [or Supporting a Claim by Another for] Small Entity Status" form [e.g. for Independent Inventor, Small Business Concern, Nonprofit Organization, Individual Non-Inventor].